World Health Organization

STANDARD OPERATING PROCEDURE

NEUROVIRULENCE TEST OF TYPES 1, 2 OR 3 LIVE ATTENUATED POLIOMYELITIS VACCINES (ORAL) IN TRANSGENIC MICE SUSCEPTIBLE TO POLIOVIRUS

Version 8
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1. Introduction

The monkey neurovirulence test for monovalent oral polio vaccine (OPV) bulks has been used, in various formats, for over 50 years. While this *in vivo* test has remained an important component of OPV quality control, the use of non-human primates has become increasingly difficult in many parts of the world. In the 1980s transgenic mice carrying the human poliovirus receptor were developed, and in 1992 WHO initiated a project to evaluate the suitability of transgenic mice for OPV neurovirulence testing. Initial studies with Serotype 3 monovalent bulks were encouraging and a series of international collaborative studies were carried out in the late 1990's to validate the design and operation of a transgenic mouse neurovirulence test (TgmNVT), first for Serotype 3, and subsequently for Serotypes 1 and 2 vaccines. The transgenic mouse test for all three poliovirus types is now part of the WHO recommendations for the production and control of OPV*(1)*.

The test is based on behavioral ("clinical") observations of groups of TgPVR21 transgenic mice inoculated intraspinally with either a test vaccine or a standardized WHO reference material (*2*). Using the standardized procedures described in this SOP mice are scored as "normal" or "paralysed," the differences between groups analysed statistically, and test vaccines that are significantly more neurovirulent than the WHO reference vaccine are rejected. The experimental design requires 32 mice (16 male and 16 female) at each dose level for each vaccine. Up to two test vaccines may be included in a single test, which may be split over two days. A total of 128 mice (one test vaccine) or 192 mice (two test vaccines) are required. The design is balanced over vaccines, doses, gender, and where appropriate, days. Randomization of the allocation of mice to vaccines and doses, the cage locations, and the order of inoculations is essential as is the blinding of these allocations to both inoculators and scorers until the completion of the test. A detailed method of statistical analysis is defined, along with associated validity tests. In an analogous manner to the monkey NVT, there is the possibility of retesting built in to decision procedure.

The Standard Operating Procedure presented here describes the currently recommended procedures for the conduct of this test for all three oral polio vaccine serotypes. It also confirms the recommendation from 1999 *(1)* that a standardized approach be taken for the qualification of institutions that wish to adopt the test for use in quality control. The recommended training program for the intraspinal inoculation technique is described in Appendix 2. After completion of the training program laboratories are then required to complete a standard implementation procedure to demonstrate their overall competence to perform the TgmNVT. Only after completion of the training and implementation process should a laboratory consider the use of batch release testing using the TgmNVT. The final decision relating to whether a laboratory can or cannot use the TgmNVT for the control of poliovirus monovalent bulks used in OPV production resides with the National Control Authority *(1)*.

Additional information on reagents, training, and contacts relating to the TgmNVT are available on the WHO web site https://www.who.int/health-topics/biologicals#tab=tab_1 or by contacting WHO at:

Unit Head, Technologies Standards and Norms (TSN)
Essential Medicines and Health Products
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20, Avenue Appia

CH-1211 Geneva 27, Switzerland

2. Transgenic mice

Only an approved strain of mice obtained from a WHO approved source should be used. At present the only approved strain is the TgPVR21 mouse line developed in Japan *(1)*. Procedures and standards for maintenance and distribution of transgenic mice susceptible to human viruses described in national or international guidelines should be observed *(3)*, and particular notice should be taken of the containment requirements that will be necessary once endemic transmission of wild poliovirus strains has been successfully ended *(4)*. The animal facility should be approved for this purpose by relevant national authorities before work with TgPVR21 commences, with copies of the documentation relating to the approval of the facility made available to the supplier and/or shipping agent if required.

3. Titration of vaccines in cell culture and preparation of inocula

3.1 Titrations of vaccines in cell culture

The recommended method for the titration of viruses is outlined in the WHO Recommendations to Assure the Quality, Safety and Efficacy of Live Attenuated Poliomyelitis Vaccine (oral) *(1)*. Briefly, this method is based upon a determination of the cell culture infectious dose ($CCID_{50}$) in Hep2C cell cultures. For estimation of the titre of any sample, replicates should be performed to obtain a precision of ±0.3 $\log_{10}$ $CCID_{50}$/ml or better for the 95% confidence limits of the mean. The dilutions of vaccine may be made in advance and aliquoted in multiple containers which should be stored at ≤ –70° C.

Titres of the diluted samples should preferably be determined by titration before the test is started to ensure that the doses to be given are correct. The required doses for each virus serotype tested against the WHO (SO+2) reference virus are:

	Upper Dose ($\log_{10}$ $CCID_{50}$/5µl)	**Lower Dose ($\log_{10}$ $CCID_{50}$/5µl)**
Type 1	2.75	1.75
Type 2	6.0	5.0
Type 3	4.5	3.5

One container per dose should be thawed on the day of the inoculations and used on that day only. The diluted vaccine containers should be stored on ice during the inoculation procedure. Residual inocula may be aliquoted and stored frozen until the back-titrations are carried out.

Three replicate back-titrations of the residual viral inocula should be performed after the injections of mice are completed to confirm that the required doses were tested. An aliquot of diluted vaccine stored continuously at ≤ –70°C may also be titrated in parallel, particularly if the diluted vaccines were not tested prior to inoculation of the samples.

As clinical manifestations of neurovirulence in TgPVR21 mice are strongly dose-dependent, extreme care should be taken to ensure that the dilution of vaccines is performed as accurately as possible, and for the test to be valid, the mean titer of the residual inocula should be within ± 0.3 $\log_{10}$ $CCID_{50}$/ml of the target dose.

3.2 Preparation of inocula

The vaccine and reference preparation should be diluted with a suitable diluent, such as those used in monkey neurovirulence testing. Eagle's minimum essential medium (EMEM) containing 0.14% bovine albumin and 0.22% sodium bicarbonate, and Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate have been successfully used by the National Institute for Biological Standards and Control (NIBSC), UK, and the U.S. Food and Drug Administration (FDA), respectively. Diluents have been shown to be important for obtaining consistent results, and if other diluents are chosen, they should be validated for equivalence to these reference diluents prior to use.

4. Animal procurement and randomization

TgPVR21 transgenic mice should be ordered to arrive at the testing institution at the age of five to six weeks and the randomization completed as soon as possible after the arrival of the mice. The mice should be allowed to recover from the shipping and randomization procedure for at least seven days before inoculation. To minimize the risk of cross-contamination it is preferable that animals should be inoculated within fourteen days of arrival at the facility. Aggressive mice should be housed in individual cages, kept separated throughout the duration of the test, and this exception recorded in the test record. Randomization is an important element of the TgmNVT and it is essential that the correct randomization procedure is followed. The procedures in Appendix 1 describe the method for randomizing mice by dose, cage location, and order of inoculation.

5. Intraspinal inoculation and clinical observations of mice

The local operating procedure should ensure that a clear record with traceability of the individual mice, cages, vaccine/reference and dose allocation is kept. However, the procedure should ensure that this information is unknown (blinded) to the inoculator and clinical scorer until after the completion of the full test.

5.1 Intraspinal inoculation

Prior to the inoculation mice should be marked in such a way that each individual in a cage can be identified. The use of differently colored indelible marker pens to streak the hair or tail has been found to be a successful and non-injurious method. The backs of the mice should be shaved or depilated to facilitate inoculation. Mice should then be anesthetized with an anesthetic mixture prepared on the day of inoculation; an example of an anesthetic is a mixture of ketamine (10 mg/ml final concentration) and xylazine (0.4 mg/ml final concentration) in physiologic saline which is injected intraperitoneally in volumes of 0.3 to 0.4 ml depending on the weight of the animal. Mice should be observed under anesthesia until they are immobile and lose pedal reflexes. The skin is then disinfected with alcohol or an equivalent disinfectant, gently lifted to form a “tent,” and a 1 to 2 cm longitudinal incision is made over the thoracic-lumbar regions of the spine. For inoculation, the mouse is placed prone with its abdomen draped over a test tube or other suitable object approximately 2 cm in diameter. Inoculations are performed using a microsyringe with a 25-µl volume and a custom-made 33-gauge needle, 1.25 cm long with a bevel angle of 15° and an external supporting 25-gauge cannula. Syringes and needles are sterilized by autoclaving for 20 minutes at 121°C. Before autoclaving the syringes must be treated with silicone solution (1 part of 360 medical grade silicone in 9 parts of 1,1,1, trichloroethene) to ensure smooth movement of the plunger.

The needle is inserted between the spinous processes of the last thoracic and the first lumbar vertebrae, directed cephalad at an angle of approximately 70° to the long axis of the spine, and advanced into the substance of the spinal cord. The needle is then pulled back for half of the length inserted into the spinal cord, turned 45° from the midline, and advanced again into the anterior gray matter or anterior horn. If the needle is inserted into the correct position, one or both hind legs should jerk. If there is no jerk, the needle should again be partially withdrawn, the angle of insertion slightly adjusted, and the needle again advanced until a leg jerk is observed. If the second insertion produces no jerk, the needle should be withdrawn completely and a new point of insertion selected. The number of attempted needle insertions should not exceed three. Once a jerk reaction is observed, 5 µl of the sample should be injected slowly over a period of 2 to 5 seconds to minimize pressure caused by the entry of the injection volume while avoiding mechanical trauma caused by needle movement. The injection may be conducted by either one or two persons depending on whether one person can simultaneously position the needle and push the syringe plunger with ease. Where this proves difficult, or where the number of animals with mechanical inoculation trauma is high, a second person should be employed to push the plunger while the needle is held firmly in place by the first. During injection of the test material mice should exhibit jerks of the hind legs, and in some cases, twisting of the rear half of the body may be observed. Following inoculation the skin incision should be closed with a suitable tissue adhesive or by an alternative means, and ophthalmic ointment applied to each eye to prevent desiccation of the cornea. It is recommended that the same inoculator performs the injections for a whole test. If injections are performed over multiple days the same inoculator should perform the injections over the whole day.

5.2 Clinical Observations

Animals should be observed for neurological signs of disease at least once per day for 14 consecutive days after inoculation and the clinical stage recorded for each observation. An additional observation 8 hours after inoculation may be conducted optionally, particularly for tests of Serotype 1 vaccines (see below). Persons inoculating mice or assigning clinical stage scores are to be blinded (unaware) of which test vaccine or dose has been administered to an animal to prevent observational bias. Four stages of clinical symptoms are defined based on characteristic motor signs:

Stages	Physical signs		
Normal	Grips the edge of the cage	Walks normally on the grid and on a flat surface	Full ability to move limbs forward
Weak	Unable to grip the edge of the cage	Walks normally on a grid or flat surface	Full ability to move limbs forward
Paresis/Partial paralysis	Unable to grip the edge of the cage	Limb falls through the grid more than once while walking steadily forward and/or toes curl repeatedly while walking on a flat surface	At least a partial ability to move limb forward
Paralysis	Unable to grip the edge of the cage	No use of limb on grid or flat surface	Inability to move the limb forward

The above 4 stages may be noted using a code devised by the observer but the code should be consistent and clearly identified on the score sheet.

For clinical scoring the examination of each mouse shall follow a step by step process:

1. The general state of the mice in the cage is observed to note possible morbidity, sickness, or injury due to aggressive behavior.
2. Weakness in the hind legs (where it often appears first) is tested by holding the mouse by the tail head down with its feet against the vertical wall of the cage. When a normal mouse is then raised, both hind limbs will instinctively grip the upper edge of the cage wall firmly. Weakness is defined by a failure of one or both hind feet to grip the cage edge. Weakness of the front limbs can be assessed by placing the front paws of the mouse on a cage lid or other similar surface and checking for resistance (firm gripping) when the animal is gently pulled or lifted by the tail. If all limbs are able to grip, the mouse can be scored as normal. If any limb is unable to grip, or if the score is questionable, the observations described in step 3 should be carried out.
3. The mouse should be observed walking. During the observation the mouse may be held by the tail but should be able to move in an unrestricted manner. A deficit in moving one or more limbs forward, a failure to raise the heel while walking, or repeated curling-under of the toes as the limb moves forward should be scored as paresis. Paralysis is scored when one or more limbs are dragged as the animal moves forward. If necessary the mouse may be made to walk on a grid (cage lid or similar surface) to aid in distinguishing the difference between weakness and paresis. If no limb passes through the grid as the animal is moving forward it can be scored as weak.

In borderline cases where it is difficult to decide between clinical stages, the observer should assign the lower score. Scores should be recorded for each observation. The score sheet should also note in which limb the symptom is observed to insure consistency in reporting.

In tests on serotypes 1 and 3 a steady progression is generally observed through weakness to paresis and paralysis. For serotype 2 however mice may show partial recovery where paralysis reverts to paresis or paresis to weakness. It is therefore essential that daily observations be conducted.

Only the advanced motor disorders of paresis and paralysis can be considered as reliable stages for determining the acceptability of a test vaccine. Therefore at the end of the experiment, animals should be scored as either Normal or Paralyzed (0 or 1). Paralyzed (1) animals are defined as those that:

1) Showed a paralysis clinical score in any limb, or
2) Showed a paresis clinical score on 2 consecutive days. Under normal practices, scoring is conducted every 24 hours, and thus two consecutive days will be equivalent to two consecutive scores.

For ethical reasons, animals which are scored as paresis on 2 consecutive days or paralysis for one day may be euthanized. Death by euthanasia in this case should be noted on the data sheet in addition to the clinical stage and the mouse will be scored as paralyzed (1) in the final score. All other surviving mice are to be euthanized on day 14.

Animals exhibiting any of the following criteria should be recorded but should be excluded from the final analysis:

1) Death due to anesthesia, normally resulting in the animal not recovering from inoculation;
2) Death due to inoculation trauma, including euthanasia of severely traumatized animals;
3) Animals that show traumatic paresis/paralysis that appears within 24 hours after the inoculation where the paralysis does not progress. This is not applicable in cases where the animal recovers after the initial trauma. In this case the animal is scored according to the normal procedure.
4) Death as a direct result of fighting or injury;
5) Other non-identified causes of death where a relationship with poliovirus clinical progression is not evident;

If more than 5% of animals are excluded for the reasons noted above the test should be repeated. It should be noted that with Serotype 1 a more rapid onset and progression of paralysis compared to the other two serotypes has been observed infrequently, making a differentiation between traumatic injury and poliovirus-related clinical signs more difficult. To monitor this, at the discretion of the testing institution mice may be additionally scored at 8 hours and 24 hours after inoculation so that this rapid progression of symptoms can be identified. This can also be performed with tests with Serotype 2 and 3 vaccines. Under blinded conditions this would require scoring all animals in the test. When animals die without any apparent cause, depending on the physical condition of the mouse body and the presence of the observer at animal death or immediately after, a post-mortem examination should be conducted by a qualified person to rule out poliovirus-related causality. Additional controls such as histological examination of the spinal cord or brain stem, or PCR testing of heparinized blood for the presence of poliovirus may be performed at the discretion of the testing institution. If frequencies of >5% inoculation trauma occur, a thorough investigation into the possible causes should be conducted and corrective actions taken.

6. Statistical analysis and decision model

The analysis of the TgmNVT is based on a comparison of the proportion of mice paralysed at each dose of the reference and test vaccines. The current WHO reference vaccines are representative of vaccine lots studied in clinical trials and found acceptable for the purpose of immunization. If a test vaccine is equally or less paralytic (neurovirulent) than the reference vaccine it is considered acceptable. If it is more paralytic it is unacceptable. In the event where a clear statistical decision is not possible, the possibility of a retest is included in the procedure.

The TgmNVT is analysed by a logistic model relating the observed paralysis proportion to the vaccine, the dose, and the gender. The relative neurovirulence of the test vaccine compared to the WHO reference vaccine is given by the "odds ratio," a number that represents how much more likely it is for paralysis to occur with the test vaccine than with the reference vaccine. An odds ratio of one indicates that the test vaccine and reference vaccine have equal neurovirulence. High values of the odds ratio indicate that a test vaccine is more neurovirulent than the reference vaccine. The log of the odds ratio (LOR) is calculated, and compared to a pre-determined L-value (analogous to the C-values in the monkey NVT). L1 and L2 values are the acceptance and rejection limits calculated from the probabilities that differences in scores are due to chance alone. If the observed LOR is less than the L1 value, the vaccine passes. If the LOR is above the

L2 value the vaccine fails. If the LOR falls between the L1 and L2 values then a retest is required to make the decision. The L-values are determined by the expected proportions of mice to be paralysed with the reference vaccine. Laboratories should generate their own L-values following the training and implementation procedure, and update them as they acquire data from further tests. L-values should cover all tests conducted by the laboratory, but to detect variability between operators, laboratories may wish to additionally calculate L-values for individual operators.

The number of mice required for the TgmNVT was determined through the original collaborative studies to ensure high probability of failing test vaccines with a specific level of neurovirulence.

The statistical analysis includes validity criteria such as a test for parallelism of dose response curves (the "Vaccine x Dose interaction"). A step by step decision procedure is defined to check the validity criteria, and come to a pass/fail decision. Provision exists for retests, either because the test is statistically invalid, or because the LOR falls between the L1 and L2 values. The decision procedure has been designed to reduce the need for retesting (and animal usage) in situations where the test is statistically non-parallel, but a decision on the test vaccine can still be made. For example, if the test vaccine is highly neurovirulent compared to the reference at both doses, it can be considered a fail even if the dose-response lines are non-parallel.

A detailed description of the statistical model and the decision procedure is given in Appendix 4. Analysis will require a suitable statistical software package for fitting the logistic regression model, and collaboration with a qualified statistician may be advisable to ensure the correct analysis and interpretation of test results.

Appendix 1: Randomization procedures for the Transgenic Mouse Neurovirulence Test (TgmNVT)

Introduction

The recommended WHO procedure for testing vaccines in the transgenic mouse neurovirulence test (TgmNVT) requires randomization of the mice to the doses, randomization of the cage locations and randomization of the order of inoculation. A randomization process is provided below for the following three cases which may be encountered during training and implementation procedures as well as during batch release testing. Calculation spreadsheets to perform the required randomization procedures are available from NIBSC on request.

- **Case 1**: one test vaccine and one reference vaccine, each at two doses, male and female mice caged separately with 4 mice per cage, 8 mice per gender-dose group, inoculation on one day. A total of 64 mice are used for this test.
- **Case 2**: one test vaccine and one reference vaccine, each at two doses, male and female mice caged separately with 4 mice per cage, 16 mice per gender-dose group, inoculation usually only on a single day. A total of 128 mice are used for this test.
- **Case 3**: two test vaccines and one reference vaccine, each at two doses, male and female mice caged separately with 4 mice per cage, 16 mice per gender-dose group, inoculation carried out over the course of two days. A total of 192 mice are used for this test.

General Principles of the Randomization

Full randomization is an important element of the TgmNVT and the random assignment of animals to dose, cage location, and order of inoculation should be assured at the onset of the test. However, the randomization procedure does not generate a complete randomization for each of combination of factors and is actually a "balanced randomization." These procedures aim to provide:

- randomization of mice to doses,
- randomization of cage locations and
- randomization of order of inoculation.

The randomization procedure can be clarified by examining Case 1 below (Tables Q1, 2 and 3), a 64 animal test with 4 doses, i.e. 8 animals per sex/dose group.

When starting the randomization, the male mice are randomized first. There will eventually be 8 cages of male mice and so Column 1 of Table Q1 distributes the first 16 mice in the first 4 cages – with one cage for each dose. The next 16 mice are distributed into the next 4 cages – again 1 cage per dose. Likewise with the females (Table Q2), four cages of females from the first 16 and another 4 from the second 16.

When the male cages are randomized in the cage location (Table Q3), the first four cages are in the first row and the second 4 in the second row. Likewise with the females, the first 4 cages in Row 1 and the second 4 in Row 2.

The following outlines some of the features of the randomization:

1. Individual mice are randomly assigned to doses/vaccines, so that the experimental unit is the mouse rather than the cage. This is preferred over randomizations of cages to doses/vaccines because it is generally more efficient and it allows analysis to be carried out on individual mouse observations instead of the more complex analysis based on cage scores.
2. Doses, vaccines and gender are not confounded with each other or with any other factors such as order of inoculation, cage location, or weight classifications. That is, statistically valid estimates and tests can be carried out for dose effects, vaccine differences, gender differences, Vaccine x Dose interactions, Vaccine x Gender interactions and Dose x Gender since they are not inextricably mixed up with each other or with other factors which may affect response.
3. A restricted randomization is applied to order of inoculation. This should provide adequate protection against bias or variance changes which may be associated with order effects. A complete randomization of inoculation order was viewed as impractical and may induce errors in experimental procedures.
4. If shipment boxes have mice grouped according to gender, weight or age (i.e. factors which are either known or suspected to affect response) the design retains this homogeneity in assignments of mice to doses/vaccines (provided that the numbers of mice within the homogeneous subgroups are consistent with the study design). That is, the randomization is equivalent to randomly assigning doses/vaccines to mice within blocks or strata defined by gender/weight/age groups.
5. Order of inoculation, weight/age groupings and location (rows) are at least partially confounded. That is, the effects of these factors are inextricably mixed up so that it is statistically impossible to differentiate the effects of one factor from the effects of the other two factors. Some degree of confounding is necessary because of practical, logistic and budgetary limitations and these three factors were considered to be of lower interest than factors such as dose, vaccine and gender.

Case 1:
The randomization process for assigning mice to doses and for assigning cage locations is provided in detail below. The randomization of the order of inoculation is accomplished by the randomization of cage locations and the specified inoculation instructions. The instructions for Case 1 are based on the randomizations provided in Tables Q1, Q2 and Q3. For each new experiment the first step is to generate a new set of Tables Q1, Q2 and Q3 as follows:

- Randomly permute (i.e. randomly re-order) the assigned cages in **each column** of Table Q1. The entries in each column of the new Table will be the same as in Table Q1 but the order will be different.
- Randomly permute the assigned cages in **each column** of Table Q2.
- Randomly permute the cage designations in **each row** of Table Q3.

After generating the new tables follow the steps outlined in the sections “Randomization Procedure” and “Inoculation Procedures” shown below.

Table Q1

Column 1		Column 2	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
1	Dm4	17	Am5
2	Bm2	18	Cm7
3	Cm3	19	Am5
4	Bm2	20	Dm8
5	Cm3	21	Dm8
6	Am1	22	Am5
7	Dm4	23	Dm8
8	Am1	24	Cm7
9	Cm3	25	Bm6
10	Bm2	26	Bm6
11	Dm4	27	Cm7
12	Am1	28	Am5
13	Dm4	29	Dm8
14	Am1	30	Bm6
15	Bm2	31	Cm7
16	Cm3	32	Bm6

Table Q2

Column 1		Column 2	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
33	Af9	49	Bf14
34	Df12	50	Df16
35	Bf10	51	Af13
36	Af9	52	Bf14
37	Df12	53	Af13
38	Af9	54	Bf14
39	Af9	55	Bf14
40	Cf11	56	Af13
41	Bf10	57	Df16
42	Df12	58	Af13
43	Cf11	59	Cf15
44	Cf11	60	Cf15
45	Bf10	61	Df16
46	Cf11	62	Cf15
47	Df12	63	Cf15
48	Bf10	64	Df16

Table Q3

Row 1	Bm2	Am1	Df12	Af9	Cm3	Bf10	Dm4	Cf11
Row 2	Bf14	Cm7	Am5	Af13	Cf15	Df16	Bm6	Dm8

Randomization Procedure

Step 1. The mice will be housed 4/cage with males and females in separate cages. The cages will be labeled as follows:

Male Mice				Female Mice			
Am1	Bm2	Cm3	Dm4	Af9	Bf10	Cf11	Df12
Am5	Bm6	Cm7	Dm8	Af13	Bf14	Cf15	Df16

That is, cages are numbered from 1 to 16, with numbers 1-8 for males (coded ‘m’) and 9-16 for females (coded ‘f’); the letters A, B, C and D indicate the coded (blinded) dose/vaccine which will be inoculated into the mice within the cage.

Step 2. It is assumed that mice are delivered to the lab in boxes of 18-20 mice which are of the same gender and similar weight/age. Arrange the shipment boxes in the following order: start with the boxes containing heavier males, followed by boxes containing lighter males, followed by boxes containing heavier females and then lighter females. In many instances there are no differences in the age/weight of animals in each of the shipping boxes, in this instance just arrange the boxes in a random order i.e. as they come to hand, with males first and females second.

Step 3. Arrange cages Am1 Bm2 Cm3 and Dm4 in a convenient manner to receive mice. Take 16 mice from the first shipment box and place them into these cages in the sequence provided in the first column of Table Q1. That is, the first mouse that comes to hand is placed in cage Dm4; the second mouse is placed in cage Bm2, the third mouse in cage Cm3, etc., the 16th mouse in cage Cm3. Cages Am1 Bm2 Cm3 and Dm4 now have 4 mice/cage.

Step 4. Arrange cages Am5 Bm6 Cm7 and Dm8 to receive the next 16 mice. As mice come to hand place them in these cages in the sequence provided in the second column of New Table Q1.

Step 5. Repeat Steps 3-4 for the female mice using cages Af9 Bf10 ... Df16 and the caging sequence provided in Table Q2.

Step 6. Place the cages in racks in the positions identified in Table Q3.

Inoculation Procedures

Step 7. Prior to inoculation the mice should be weighed. Gender, weight/age, cage identification, location, dose/vaccine, and outcome (paralysis) must be recorded for each mouse. Records must also be maintained of the date mice arrive at the lab, date of caging and inoculation, results of back-titration, any losses and reasons for the losses (e.g. accidental deaths) and any deviation from protocol (e.g. individual housing of overly aggressive mice).

Step 8. Randomize the doses to be used in the inoculation and assign them the letters A, B, C and D. To minimize bias in assessing behavioral clues during inoculation and scoring, neither the inoculator(s) nor clinical scorer(s) should be made aware of which vaccine or dose corresponds to which letter until data collection is complete.

Step 9. Starting with the cage at the left end of Row 1 (see Table Q3), inoculate the 4 mice in this cage with the dose/vaccine specified on the cage. Then inoculate the mice in the next cage of Row 1, etc until Row 1 is completed. Proceed to Row 2 starting at the left and complete inoculations for Row 2. All inoculations are to be performed by one person on a single day.

Case 2:

This case relates to a test of vaccines versus reference with 16 animals per gender/dose group i.e. a total of 128 mice. The instructions for Case 2 are based on the randomizations provided in Tables D1, D2 and D3.

For each new experiment the first step is to generate a new set of Tables D1, D2 and D3 as follows:

Table D1

Column 1		Column 2		Column 3		Column 4	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
1	Dm4	17	Am5	33	Am9	49	Bm14
2	Bm2	18	Cm7	34	Dm12	50	Dm16
3	Cm3	19	Am5	35	Bm10	51	Am13
4	Bm2	20	Dm8	36	Am9	52	Bm14
5	Cm3	21	Dm8	37	Dm12	53	Am13
6	Am1	22	Am5	38	Am9	54	Bm14
7	Dm4	23	Dm8	39	Am9	55	Bm14
8	Am1	24	Cm7	40	Cm11	56	Am13
9	Cm3	25	Bm6	41	Bm10	57	Dm16
10	Bm2	26	Bm6	42	Dm12	58	Am13
11	Dm4	27	Cm7	43	Cm11	59	Cm15
12	Am1	28	Am5	44	Cm11	60	Cm15
13	Dm4	29	Dm8	45	Bm10	61	Dm16
14	Am1	30	Bm6	46	Cm11	62	Cm15
15	Bm2	31	Cm7	47	Dm12	63	Cm15
16	Cm3	32	Bm6	48	Bm10	64	Dm16

Table D2

Column 1		Column 2		Column 3		Column 4	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
65	Af17	81	Af21	97	Af25	113	Af29
66	Cf19	82	Bf22	98	Cf27	114	Df32
67	Bf18	83	Bf22	99	Af25	115	Bf30
68	Af17	84	Df24	100	Df28	116	Af29
69	Df20	85	Cf23	101	Df28	117	Cf31
70	Bf18	86	Bf22	102	Bf26	118	Bf30
71	Cf19	87	Df24	103	Bf26	119	Df32
72	Df20	88	Af21	104	Cf27	120	Cf31
73	Bf18	89	Cf23	105	Af25	121	Cf31
74	Bf18	90	Bf22	106	Bf26	122	Df32
75	Af17	91	Af21	107	Df28	123	Bf30
76	Df20	92	Cf23	108	Bf26	124	Af29
77	Cf19	93	Af21	109	Cf27	125	Bf30
78	Cf19	94	Cf23	110	Af25	126	Df32
79	Df20	95	Df24	111	Df28	127	Cf31
80	Af17	96	Df24	112	Cf27	128	Af29

Table D3

Row 1	Bm2	Am1	Df20	Af17	Cm3	Bf18	Dm4	Cf19
Row 2	Bf22	Cm7	Am5	Af21	Cf23	Df24	Bm6	Dm8
Row 3	Cm11	Af25	Bf26	Df28	Am9	Dm12	Bm10	Cf27
Row 4	Am13	Bm14	Bf30	Af29	Dm16	Cm15	Cf31	Df32

- Randomly permute (i.e. randomly re-order) the assigned cages in **each column** of Table D1. The entries in each column of the new Table will be the same as in Table D1 but the order will be different.
- Randomly permute the assigned cages in **each column** of Table D2.
- Randomly permute the cage designations in **each row** of Table D3.

After generating the new tables follow the steps outlined in the sections "Randomization Procedure" and "Inoculation Procedures" shown below.

Randomization Procedure

Randomization for an experiment with a test vaccine and a reference vaccine, each at two doses with 16 mice per gender/dose/vaccine group.

Step 1. The mice will be housed 4/cage with males and females in separate cages. The cages will be labeled as follows:

Male Mice				Female Mice			
Am1	Bm2	Cm3	Dm4	Af17	Bf18	Cf19	Df20
Am5	Bm6	Cm7	Dm8	Af21	Bf22	Cf23	Df24
Am9	Bm10	Cm11	Dm12	Af25	Bf26	Cf27	Df28
Am13	Bm14	Cm15	Dm16	Af29	Bf30	Cf31	Df32

That is, cages are numbered from 1 to 32, with numbers 1-16 for males (coded 'm') and 17-32 for females (coded 'f'); the letters A, B, C and D indicate the dose/vaccine which will be inoculated into the mice within the cage. i.e. vaccine and reference, 2 doses of each.

Step 2. It is assumed that mice are delivered to the lab in boxes of 18-20 mice which are of the same gender and similar weight/age. Arrange the shipment boxes in the following order: start with the boxes containing heavier males, followed by boxes containing lighter males, followed by boxes containing heavier females and then lighter females. If there is no age/size difference between mice then randomly arrange male and female boxes.

Step 3. Arrange cages Am1 Bm2 Cm3 and Dm4 in a convenient manner to receive mice. Take 16 mice from the first shipment box and place them into these cages in the sequence provided in the first column of Table D1. That is, the first mouse that comes to hand is placed in cage Dm4; the second mouse is placed in cage Bm2, the third mouse in cage Cm3, etc., the 16th mouse in cage Cm3. Cages Am1, Bm2, Cm3, and Dm4 now have 4 mice/cage.

Step 4. Arrange cages Am5 Bm6 Cm7 and Dm8 to receive the next 16 mice. As mice come to hand place them in these cages in the sequence provided in the second column of Table D1.

Step 5. Repeat the Step 4 with cages Am9 Bm10 Cm11 and Dm12 using the sequence provided in the third column of Table D1.

Step 6. Repeat the Step 4 with cages Am13 Bm14 Cm15 and Dm16 using the sequence provided in the fourth column of Table D1.

Step 7. Repeat Steps 3-6 for the female mice using cages Af17 Bf18 ... Df32 and the caging sequence provided in New Table D2.

Step 8. Place the cages in racks in the positions identified in Table D3.

Inoculation Procedures

Step 9. Prior to inoculation the mice should be weighed. Gender, weight/age, cage identification, location, dose/vaccine, and outcome (paralysis) must be recorded for each mouse. Records must also be maintained of the date mice arrive at the lab, date of caging and inoculation, results of back-titration, any losses and reasons for the losses (e.g. accidental deaths) and any deviation from protocol (e.g. individual housing of overly aggressive mice).

Step 10. Randomize the doses to be used in the inoculation and assign them the letters A, B, C and D. To minimize bias in assessing behavioral clues during inoculation and scoring, neither the inoculator(s) nor clinical scorer(s) should be aware of which vaccine or dose corresponds to which letter until data collection is complete.

Step 11. Starting with the cage at the left end of Row 1 (see Table D3), inoculate the 4 mice in this cage with the dose/vaccine specified on the cage. Then inoculate the mice in the next cage of Row 1, etc until Row 1 is completed. Proceed to Row 2 starting at the left and complete inoculations for Row 2. All inoculations are to be performed by one person on a single day.

Case 3:

This case is based on a test for 2 vaccines versus the reference with 16 mice per dose/gender group. A total of 192 mice are used for this study. The instructions for Case 3 are based on the randomizations provided in Tables F1, F2 and F3. For each new experiment the first step is to generate a new set of Tables F1, F2 and F3 as follows:

- Randomly permute (i.e. randomly re-order) the assigned cages in **each column** of Table F1. The entries in each column of the new Table will be the same as in Table F1 but the order will be different.
- Randomly permute the assigned cages in **each column** of Table F2.
- Randomly permute the cage designations in **Rows 1 and 2** of Table F3 and then randomly permute the cage designations of the **Rows 3 and 4**, **Rows 5 and 6** and **Rows 7 and 8** of Table F3

After generating the new tables follow the steps outlined in the sections “Randomization Procedure” and “Inoculation Procedures” shown below.

Randomization Procedure

Randomization for an Experiment with 2 test vaccines and a reference vaccine, each at two doses with 16 mice per gender/dose group i.e. a total of 192 mice.

Step 1. The mice will be housed 4/cage with males and females in separate cages. The cages will be labeled as follows:

Male mice

Am1	Bm2	Cm3	Dm4	Em5	Fm6
Am7	Bm8	Cm9	Dm10	Em11	Fm12
Am13	Bm14	Cm15	Dm16	Em17	Fm18
Am19	Bm20	Cm21	Dm22	Em23	Fm24

Female mice

Af25	Bf26	Cf27	Df28	Ef29	Ff30
Af31	Bf32	Cf33	Df34	Ef35	Ff36
Af37	Bf38	Cf39	Df40	Ef41	Ff42
Af43	Bf44	Cf45	Df46	Ef47	Ff48

Table F1

Column 1		Column 2		Column 3		Column 4	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
1	Bm2	25	Dm10	49	Em17	73	Dm22
2	Fm6	26	Em11	50	Bm14	74	Em23
3	Dm4	27	Cm9	51	Fm18	75	Bm20
4	Am1	28	Em11	52	Cm15	76	Fm24
5	Fm6	29	Am7	53	Bm14	77	Am19
6	Cm3	30	Am7	54	Fm18	78	Em23
7	Em5	31	Fm12	55	Cm15	79	Cm21
8	Bm2	32	Am7	56	Am13	80	Bm20
9	Cm3	33	Dm10	57	Am13	81	Am19
10	Am1	34	Bm8	58	Am13	82	Bm20
11	Am1	35	Am7	59	Am13	83	Dm22
12	Fm6	36	Dm10	60	Dm16	84	Cm21
13	Em5	37	Bm8	61	Em17	85	Dm22
14	Em5	38	Fm12	62	Cm15	86	Fm24
15	Am1	39	Bm8	63	Fm18	87	Bm20
16	Bm2	40	Dm10	64	Em17	88	Fm24
17	Bm2	41	Cm9	65	Fm18	89	Cm21
18	Dm4	42	Em11	66	Dm16	90	Am19
19	Dm4	43	Fm12	67	Bm14	91	Dm22
20	Em5	44	Em11	68	Dm16	92	Em23
21	Cm3	45	Bm8	69	Dm16	93	Fm24
22	Cm3	46	Fm12	70	Em17	94	Cm21
23	Fm6	47	Cm9	71	Bm14	95	Em23
24	Dm4	48	Cm9	72	Cm15	96	Am19

That is, cages are numbered from 1 to 48, with numbers 1-24 for males (coded 'm') and 25-48 for females (coded 'f'); the letters A, B, C, D, E and F indicate the dose/vaccine which will be inoculated into the mice within the cage.

Step 2. It is assumed that mice are delivered to the lab in boxes of 18-20 mice which are of the same gender and similar weight/age. Arrange the shipment boxes in the following order: start with the boxes containing heavier males, followed by boxes containing lighter males, followed by boxes containing heavier females and then lighter females. If there is no age/size difference between mice then randomly arrange male and female boxes.

Step 3. Arrange cages Am1 Bm2 Cm3 Dm4 Em5 and Fm6 in a convenient manner to receive mice. Take 24 mice from the first 2 shipment boxes and place them into these cages in the sequence provided in the first column of Table F1. That is, the first mouse that comes to hand is placed in cage Bm2; the second mouse is placed in cage Fm6, the third mouse in cage Dm4, etc., the 24th mouse in cage Dm4. Cages Am1, Bm2, Cm3, Dm4, Em5, and Fm6 now have 4 mice/cage.

Table F2

Column 1		Column 2		Column 3		Column 4	
Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage	Mouse Handling Order	Assigned Cage
97	Cf27	121	Cf33	145	Bf38	169	Bf44
98	Bf26	122	Bf32	146	Ef41	170	Df46
99	Af25	123	Af31	147	Ff42	171	Af43
100	Df28	124	Df34	148	Df40	172	Af43
101	Cf27	125	Cf33	149	Bf38	173	Df46
102	Ff30	126	Ff36	150	Cf39	174	Ef47
103	Ef29	127	Ef35	151	Ff42	175	Ff48
104	Df28	128	Df34	152	Cf39	176	Bf44
105	Cf27	129	Cf33	153	Cf39	177	Ff48
106	Ff30	130	Ff36	154	Df40	178	Ff48
107	Cf27	131	Cf33	155	Af37	179	Ef47
108	Df28	132	Df34	156	Bf38	180	Ef47
109	Bf26	133	Bf32	157	Df40	181	Ef47
110	Ff30	134	Ff36	158	Df40	182	Cf45
111	Df28	135	Df34	159	Bf38	183	Cf45
112	Ef29	136	Ef35	160	Ef41	184	Df46
113	Ef29	137	Ef35	161	Af37	185	Bf44
114	Bf26	138	Bf32	162	Af37	186	Ff48
115	Af25	139	Af31	163	Af37	187	Cf45
116	Ef29	140	Ef35	164	Ff42	188	Af43
117	Af25	141	Af31	165	Ef41	189	Af43
118	Bf26	142	Bf32	166	Ef41	190	Bf44
119	Af25	143	Af31	167	Cf39	191	Cf45
120	Ff30	144	Ff36	168	Ff42	192	Df46

Step 4. Arrange cages Am7 Bm8 Cm9 Dm10 Em11 and Fm12 to receive the next 24 mice. As mice come to hand place them in these cages in the sequence provided in the second column of Table F1.

Step 5. Repeat Step 4 with cages Am13 Bm14 Cm15 Dm16 Em17 and Fm18 using the sequence provided in the third column of New Table D4.

Step 6. Repeat the Step 4 with cages Am19, Bm20, Cm21, Dm22, Em23, and Fm24 using the sequence provided in the fourth column of New Table D4.

Step 7. Repeat Steps 3-6 for the female mice using cages Af25, Bf26, Cf27 .... Ff48 using the caging sequence provided in Table F2.

Step 8. Place the cages in racks in the positions identified in Table F3.

Table F3

Row 1	Df28	Bf26	Ff30	Dm4	Am1	Bm2
Row 2	Em5	Cf27	Cm3	Af25	Fm6	Ef29
Row 3	Bm8	Df34	Cf33	Af31	Fm12	Bf32
Row 4	Ff36	Ef35	Em11	Dm10	Cm9	Am7
Row 5	Ef41	Bm14	Cm15	Cf39	Bf38	Fm18
Row 6	Af37	Em17	Df40	Am13	Dm16	Ff42
Row 7	Df46	Bf44	Fm24	Af43	Ef47	Em23
Row 8	Cm21	Cf45	Ff48	Bm20	Am19	Dm22

Inoculation Procedures

Step 9. Prior to inoculation the mice should be weighed. Gender, weight/age, cage identification, location, dose/vaccine, and outcome (paralysis) must be recorded for each mouse. Records must also be maintained of the date mice arrive at the lab, date of caging and inoculation, results of back-titration, any losses and reasons for the losses (e.g. accidental deaths) and any deviation from protocol (e.g. individual housing of overly aggressive mice).

Randomize the doses to be used in the inoculation and assign them the letters A, B, C, D, E and F. To minimize bias in assessing behavioral clues during inoculation and scoring, neither the inoculator(s) nor clinical scorer(s) should be aware of which vaccine or dose corresponds to which letter until data collection is complete.

Step 11. Starting with the cage at the left end of Row 1 (see Table D3), inoculate the 4 mice in this cage with the dose/vaccine specified for the cage. Then inoculate the mice in the next cage of Row 1, etc until Row 1 is completed. Proceed to Row 2 starting at the left and complete inoculations for Row 2.

Step 12. Inoculation for this study can be completed in a single day; however, it is generally found that it is more appropriate to split the inoculations across 2 days. If this is to be done then:

- Rows 1-4 should be inoculated on Day 1 and Rows 5-8 on Day 2.
- A separate vial of each of the six samples should be used on each of the days that inoculations are carried out.
- Either one inoculator should perform all injections over two days, or if two inoculators are needed, all injections on a day should be performed by the same inoculator.

Appendix 2: Recommended training program for the Transgenic Mouse Neurovirulence Test (TgmNVT) and for the training of new inoculators and maintenance of competence of qualified inoculators

Introduction

The initial training program for an inoculator/organization consists of three steps. Each consecutive step should be made only after the previous step has been accomplished.

The Training Steps

Step 1. Obtain training in the technique of intraspinal inoculation of mice at a WHO recognized training laboratory. If the organization has not obtained training in the clinical scoring of the TgmNVT they should also be trained in this area during Step 1.

Step 2. Inoculate conventional mice intraspinally with 5 µl of 10% India ink, dissect out the spinal cord and examine for the presence of the ink in the correct region of the cord.

Step 3. Conduct two independent tests in TgPVR21 mice for each of Serotypes 1, 2 and 3. An organization need only complete the training tests for each serotype once. Training of new inoculators will involve a reduced set of tests designed to demonstrate the competence of the inoculator to perform the procedure rather than demonstrate the ability of the organization to undertake the test in full.

Preliminary Training- Step 1

Training for Inoculators

WHO can provide information on laboratories that will be able to assist in the preliminary training phase for an organization undertaking the training and implementation for the first time.

Training in clinical scoring

Each of the three serotypes can manifest subtly different clinical symptoms and it is therefore important that an organization undertaking training in clinical scoring attempts to observe and score tests of all three serotypes during the training phase. The suggested procedure for training in the clinical scoring is:

1) An observation/explanation phase with a qualified operator (usually 1 or 2 tests),

2) Assisted scoring sessions where the qualified operator and the trainee score tests together (usually 1 or 2 tests),

3) At this stage the trainee and the trainer score the same animals independently in a mock test situation (i.e. a full scoring day). The scoring should include at least forty animals with clinical signs of polio infection. Timing between the scoring by the trainer and the trainee should be minimized to reduce the possibility that the disease will progress between scoring sessions. The trainer should always score the animals before the trainee. At the end of scoring the score sheets are compared. Any differences are confirmed in a second reading by the trainer and where appropriate the corrected scores are used to calculate the concordance level. To be considered competent the result of the trainee's scoring should be evaluated in comparison to the trainer's for the number of scoring difference (D) over the total number of animals (N) (D/N) and the number of scoring differences involving the change from weak to partial paralysis 1 (Dw-pp) over the number of animals with clinical signs (CS) (Dw-pp/CS). For D/N and Dw-pp/CS the trainee should not differ more than 5% from the trainer's score. If the trainee does not meet the established criteria they should begin again with at least step 2 of the procedure. If the exercise must be repeated again due to an unsatisfactory score, only the most recent data is considered in the next calculation of the scoring difference.

A trainee should also be able to adequately evaluate the inoculation step (including the evaluation of the documentation related to randomization of mice, tracking of inocula and mice/cages etc.) and perform the calculations of the results. Appropriate training data sets for calculation and example documentation on inoculation should be used to ensure that the correct results are obtained by the trainee. (NIBSC is willing to provide the appropriate training data sets if requested by the OMCL). NIBSC is willing to act as the training centre for any of the three stages noted above upon arrangement with the interested OMCL and could if needed provide a letter indicating that the step(s) had been completed successfully.

In preparation for the hands on training, in a preliminary phase, the trainee should watch the training DVD on clinical evaluation of oral polio vaccine testing in transgenic mice prepared by and available from NIBSC.

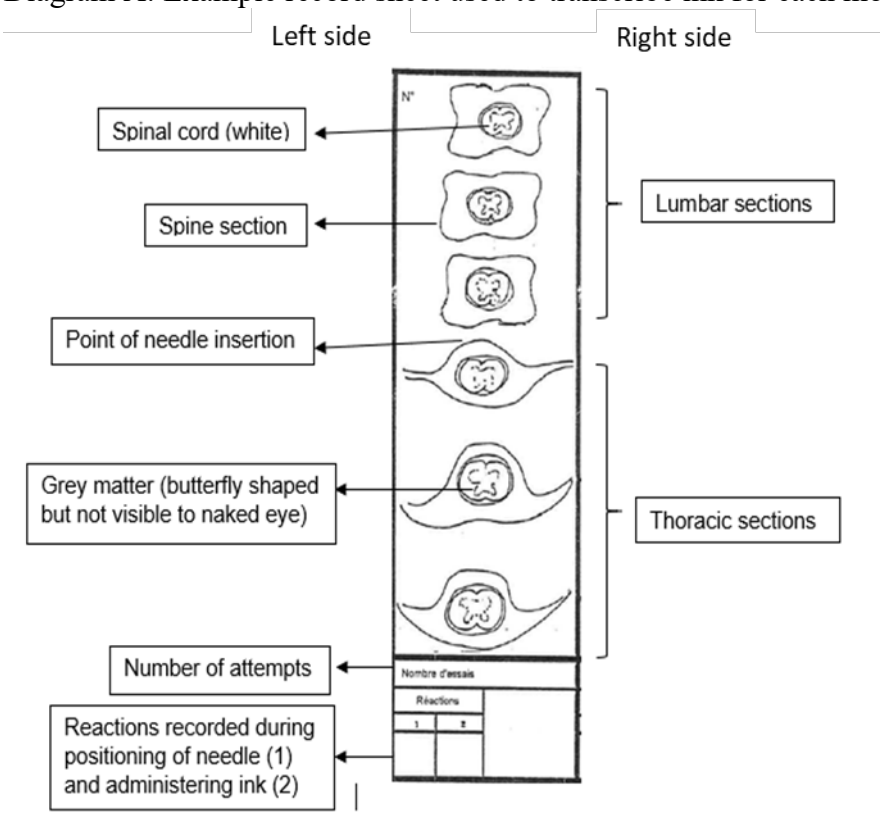
It is recommended that the above training all be completed within a 6 month period and that adequate records be kept of all training sessions.

Within an organization regular parallel scoring sessions should be undertaken as described above to ensure that members of staff are consistent and agree on the clinical criteria to be applied. It is also recommended that whenever possible organizations conduct training in clinical scoring with other organizations to avoid shifts in interpretation and application of the criteria that can occur over time. In the case of a long period of scoring inactivity (greater than 6 months) measures should be taken to re-qualify observation skills before a test scoring session is undertaken.

Dye Inoculation –Step 2

Mice are inoculated using the same procedure as the standard test, but instead of vaccine, a 10% India ink solution is injected to check placement of the needle and assess the extent of diffusion of injected material. Mice are euthanized immediately after inoculation and the spinal column removed and fixed in 10% formalin for 2 days. Alternatively the spine can be frozen and several blocks of the frozen spinal column prepared using a sharp blade. The spine is positioned with the low thoracic region nearest to the operator and cut transversely through the point of insertion visible as a black dot (between the last thoracic and the first lumbar vertebrae). Then at least three sections are transversely sliced from the low thoracic region and at least three sections from the lumbar region, as thinly as possible (1-2mm). The sections are laid with the cranial side uppermost, and the blocks examined. All visible ink on the consecutive sections is transcribed onto record sheets (see diagram A). The number of attempts and reactions observed in hind limb(s) during injection is recorded for each injection. Each test should consist of at least 30 animals.

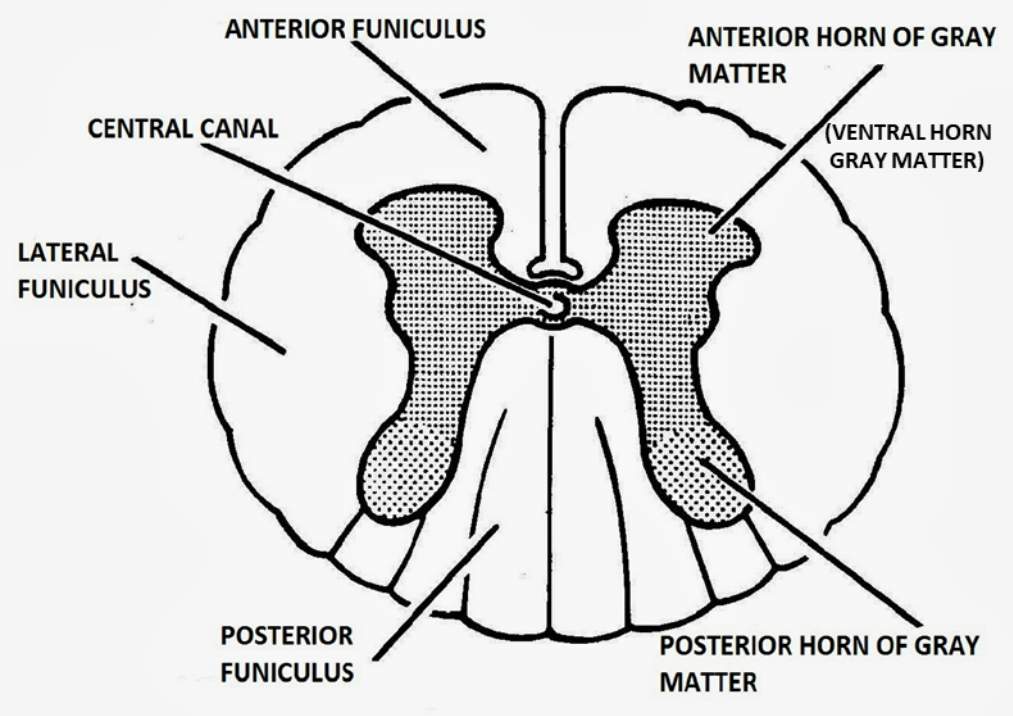
Diagram A: Example record sheet used to transcribe ink for each mouse/spine



Animals have been correctly inoculated if there is an India ink deposit in the anterior gray matter (ventral horn gray matter) (see diagram B) . An inoculator is considered competent to pass to the next stage of the training if ≥ 90% of animals are inoculated correctly in each of 3 consecutive tests.*

* There is no general rule about how many mice and how long it may take for any given inoculator to become competent i.e. to achieve >90% accuracy in 3 consecutive tests. However, with regular training sessions (at 2-4 week intervals) and adequate numbers of mice in each session (30 or more) experience indicates that the desired accuracy over three consecutive tests may require up to 20 tests.

Diagram B: Anatomy of spinal cord



From experience the following can occur:

- When the left hind limb reacts during inoculation, ink is visible in the left-hand side anterior grey matter and vice versa for the right hind limb.
- Ink should be visible in a similar position in adjacent sections.
- A second reaction of the hind limb is not always noted but the injection is still recorded as successful if the ink dictates.
- The pen used to record the ink spot should allow to reproduce precisely the ink spot observed.
- Ink observed in a vertical midline or adjacent to the central canal does not dictate a successful inoculation.

The Training Tests – Step 3

Serotype 1

Training for Type 1 involves two separate tests of a Type 1 preparation alongside the WHO/I reference preparation. The dilutions producing the required doses for both vaccine and reference will be given in the documentation provided with the samples.

Dilutions are made as described in the relevant documentation and the design and randomization procedure is as provided in Appendix 1, Case 2 with 16 mice/dose-gender. Each study must satisfy validity criteria c1, c2, c3:

c1 The WHO/I reference preparation paralysis rates at doses 1.75 and 2.75 must be between 0.05 and 0.95;

c2 The WHO/I reference vaccine must have a statistically significant ($p<0.05$, one-tailed test) increase in paralysis rates from the 1.75 to the 2.75 dose;
c3 The test for Vaccine x Dose interaction must have a p-value >0.05 (i.e. no evidence of a Vaccine x Dose interaction).

Serotype 2

Training for type 2 involves two separate tests of a single vaccine and the WHO/II reference preparation. The dilutions producing the required doses for both vaccine and reference will be given in the documentation provided with the samples.

Dilutions are made as described in the relevant documentation and the design and randomization procedure is as provided in Appendix 1, Case 2 with 16 mice/dose-gender. Each study must satisfy validity criteria c1, c2, c3:

c1 The WHO/II reference vaccine paralysis rates at doses 5.0 and 6.0 must be between 0.05 and 0.95;
c2 The WHO/II reference vaccine must have a statistically significant ($p<0.05$, one-tailed test) increase in paralysis rates from the 5.0 to the 6.0 dose;
c3 The test for Vaccine x Dose interaction must have a p-value >0.05 (i.e. no evidence of a Vaccine x Dose interaction).

Serotype 3

Training for type 3 requires inoculation of two different vaccines in the two tests. The first test uses a vaccine with a markedly elevated amount of a known neurovirulent mutation at position 472 of the viral genome (472-C revertants). This sample is tested alongside the WHO/III reference preparation. The dilutions producing the required doses for both vaccine and reference will be given in the documentation provided with the samples.

The design and randomization procedure is as provided in Appendix 1, Case 1 with a reduced number of mice (8 mice/dose-gender) as this vaccine should be a clear fail in the test. The study must satisfy the following validity criteria:

c1 The WHO/III reference vaccine paralysis rates at doses 3.5 and 4.5 must be between 0.05 and 0.95;
c2 The WHO/III reference vaccine must have a statistically significant ($p<0.05$, one-tailed test) increase in paralysis rates from the 3.5 to the 4.5 dose;
c3 The decision model applied to the paralysis data must result in rejection of the test vaccine.

If this test is satisfactory a second test is carried out with a vaccine with a moderately elevated amount of 472-C revertants and the WHO/III reference vaccine. The design and randomization procedure provided in Appendix 1, Case 2 with 16 mice/dose-gender should be followed. The study must satisfy validity criteria c1, c2 and c3 in Step 4 and the following additional criterion:

c4 The test for Vaccine x Dose interaction must have a p-value >0.05 (i.e. no evidence of a Vaccine x Dose interaction).

Return of Data

Upon completion of a test the following data should be provided to the WHO-affiliated reference laboratory. The NCL may also require submission of these data at this time or at a later stage. The following data should be included:

- Randomization scheme used showing the allocation of mice of each sex by cage number, the allocation of vaccine/dose sample by cage, the order in which cages were inoculated and any additional data e.g. dates on which each cage were inoculated;
- Daily clinical record sheets for all mice;
- Summary of the results from the test. These should be provided in a spreadsheet in which following are presented for each mouse:

Mouse Number	Sex (M/F)	Mouse weight	Cage	Sample Inoculated	Dose	Inoculator	Date Inoculated	Clinical stage (max)	Paralysis score (0/1)
1	M	28.18	Dm4	WHO/III	4.5	FB	29/2/04	paresis	1
2	….	…							

For the initial training phase for the TgmNVT the following data are not required, but may be submitted:

- Titration data for the dilutions and the titration reference prior to inoculation into mice.
- Titration data for the residual inocula.
- Decisions on validity of the test and the pass/fail outcome for each sample.

Training for New Inoculators

For organizations that have completed the implementation process for at least one virus serotype the training process for a new inoculator is:

Step 1. Obtain training in the technique of intraspinal inoculation of mice with a qualified inoculator within the organization.

Step 2. Inoculate conventional mice intraspinally with 5 µl of 10% India ink, dissect out the spinal cord and examine for the presence of the ink in the correct region of the cord. The success criteria at this step are the same as outlined above, i.e. 3 consecutive tests of 30 or more mice with an accuracy of 90% or greater.

Step 3. Training tests: only three tests with viruses from the training or implementation panels need to be completed, with the expected result being obtained in each of the three tests. The schedule for tests to be undertaken should be agreed with the WHO affiliated reference laboratory.

Step 4. The WHO affiliated reference laboratory should provide details of the test samples to the NRA and the results of the tests should be provided by the testing organization to the NRA.

Maintenance of Competence

Following qualification, inoculators need to continue inoculating to retain the competence. If a qualified inoculator has not completed a test within the previous 3 months they should validate their technique by completing a series of intraspinal inoculations with India ink. Not less than 30

mice should be inoculated and an accuracy of 90% or greater must be achieved. Failure to achieve 90% accuracy will require that they carry out the India ink inoculation phase of this training procedure until they have reached an accuracy of 90% or greater on three consecutive tests. After achieving this accuracy the inoculator can then resume TgmNVT inoculations.

Each inoculator shall perform a minimum of one India ink inoculation per year. Records of all tests carried out for maintenance of competence should be retained and made available for the NCL if requested.

DVDs with recordings of mice at the different stages of clinical signs of paralysis will be used as a teaching and monitoring aid. Two types of DVD are available:

1) **Training and refreshing observation skills**: For a novice scorer, to familiarize them with the different stages and provide harmonized examples and for experienced scorers to refresh their visual memory, especially when there has been a significant time lapse since the scorer has last evaluated a test. This style of DVD clearly identifies examples and provides commentary.
2) **PTS-like exercise**: In order to evaluate the maintenance of competence DVDs will be prepared with a compilation of images of mice at different stages. The stages of the mice will not be identified. The reader will complete a score sheet and submit it to the organizer for evaluation. If the score does not fall within a given limit of difference (to be determined based on NIBSC data score sheets from different operators) the scorer would be required to go for additional training. NIBSC has committed to preparing the DVDs for both purposes. PTS-like studies will be run at least once per year.

Appendix 3: Recommended implementation process for the Transgenic Mouse Neurovirulence Test (TgmNVT)

Introduction

Following successful completion of the "Recommended Training Program for the Transgenic Mouse Neurovirulence Test (TgmNVT)" (see Appendix 2), WHO recommends that laboratories complete the standard implementation process to validate its readiness to perform the TgmNVT on commercial vaccine batches. For this purpose the laboratory must conduct three qualifying experiments per serotype with a coded proficiency panel supplied by a WHO nominated reference laboratory. Each panel consists of six samples and two samples are tested concurrently against the homotypic WHO reference virus in each of three tests. The appropriate design and randomization protocols for these tests are provided in Appendix 1, Case 3.

Provision of Samples

Once training is successfully completed the standard implementation panel may be requested from WHO affiliated reference laboratory. For each serotype the panel consists of six coded samples that have been previously tested in both the monkey and mouse NVT. The samples are selected to provide a stringent evaluation of the performance of the mouse test. No information is provided on the titre or expected result for each sample. Laboratories are requested to test all six samples then to send their raw data and pass/fail decisions to the WHO affiliated reference laboratory for evaluation.

Titration of Samples

In parallel with the training process in mice, laboratories should document the precision and evaluate the sensitivity of their viral titration procedures for each serotype they subsequently test in the TgmNVT. Reference preparations for viral titrations can be provided by a WHO affiliated reference laboratory for this purpose. Because of the sensitivity of the mouse neurovirulence test to virus dose, laboratories should achieve for each serotype a precision of $\pm$ 0.3 $\log_{10}$ $CCID_{50}$/ml or better for the confidence limits of the mean titre. The observed titres should also be compared with the assigned titres of the titration reference preparations.

Calibration of an in-house titration reference

It is recommended that laboratories calibrate their own in-house reference against the available titration references for use in assigning titres for the TgmNVT.

Tests in mice

The implementation process requires that each sample be tested once in mice. Each test should include the WHO reference preparation plus two samples and will require 192 mice. "Instructions for Use" will accompany each panel and will explain which samples are to be included in each of the three tests.

A minimum of three tests is required to complete the implementation process for each serotype. As part of the process organizations are requested to make an assessment of the validity of each test and, for valid tests, whether each sample passes or fails. When three valid tests are completed, raw data from all tests performed in the qualification process (valid and invalid) should be forwarded to the WHO affiliated reference center coordinating the implementation process for further evaluation. The data and format to be provided will be given in the "Instructions for Use" that accompany each of the panels.

Return of Data

Upon completion of the tests for each implementation panel the following data should be provided:

- Titration data for each of the samples, the WHO (SO+2) standard, and the titration reference. Data should be provided for each titration undertaken;
- Titration data for the dilutions and the titration reference measured prior to inoculation into mice. Data should be provided for each titration undertaken;
- Titration data for the residual inocula measured after inoculation into mice. Data should be provided for each titration undertaken;
- Randomization scheme used showing the allocation of mice of each sex by cage number, the allocation of vaccine/dose sample by cage, the order in which cages were inoculated and any additional data, e.g., dates on which each cage were inoculated;
- Daily clinical scoring records for all mice;
- Summary of the results from the test. These should be provided in a spreadsheet in which data are presented for each mouse, as shown below. Additional data such as unexpected mortality should also be recorded;
- Decisions on validity of the test and the pass/fail outcome for each sample;
- Recalculated L values and the numbers used to recalculate these values.

Evaluation

The evaluation will initially involve re-assessment of whether each test meets validity criteria specified in the SOP. These are:

- that the doses of virus inoculated into mice should be within ± 0.3 $\log_{10}$ $CCID_{50}$ of the target dose;
- the paralysis proportions of the WHO reference are within limits;
- there is a significant increase in paralysis for the WHO reference from the low to the high dose;
- that there is no evidence for a Vaccine x Dose interaction. The evaluation will also compare the observed outcome (pass or fail) with the expected outcome.

In addition the data will be reviewed to determine that there are no Vaccine x Gender and Dose x Gender effects, that the estimated log odds ratios from the tests of the duplicate vaccines are not significantly different from each other, and that the vaccines are successfully ranked according to their expected neurovirulence. Any inconsistencies identified in these additional evaluations will indicate a need for careful review of procedures rather than invalidate the performance of the laboratory.

Criteria to be used to determine if a laboratory successfully completes the implementation process

Satisfactory completion of the implementation process requires that an organization completes a valid test for all six samples from a total of not more than five tests and obtains the expected results for all six samples. Failure to meet these criteria will indicate a need for retraining. The extent of the retraining shall be determined by the NRA in collaboration with the WHO affiliated reference laboratory.

Introduction of the mouse test for batch release

A laboratory that has successfully completed the implementation process should use the accumulated data from 5 valid tests (3 in this implementation process plus 2 from the training assays) to determine their own L_1 and L_2 values and use these for batch release purposes (see also

Appendix 4). The accumulated data may be used to support a regulatory application to perform the mouse TgmNVT.

Appendix 4: Statistical analysis and decision model for testing Serotype 1, 2 or 3 OPV vaccines in transgenic mice

Introduction

Logit transformations of Paralysis Proportions are represented as linear functions of vaccine, dose and study design parameters (e.g. lab, replicate within lab, inoculation day, gender). Parameter estimates and significance tests are based on Maximum Likelihood procedures and log likelihood chi-squared tests. To test an interaction, a chi-squared test is applied to the difference in log likelihoods between the main effects model with and without the interaction term. Main effects are tested in a similar manner by applying a chi-squared test to the difference in log likelihoods between the main effects model with and without the main effect under consideration. The main effects models are parameterized so that the estimates of the vaccine effects and dose effects are represented as the log odds ratios. That is, the relative neurovirulence of a test vaccine and the WHO (SO+2) reference is estimated by the log odds ratio. Estimates and standard errors derived from Maximum Likelihood analyses of the main effects models can be used to calculate 95% confidence intervals. Exponential transformation of the estimates and confidence limits provide estimated odds ratios and 95% fiducial limits for the odds ratio. The odds ratio indicates how much more likely it is for paralysis to occur in the test vaccine than in the reference vaccine.

The recommended methodology for comparing a test vaccine against the WHO reference requires:

- the test vaccine be tested concurrently with the WHO reference vaccine in a randomized experiment; and that
- both vaccines are to be tested at 2 doses, a high and low dose, as shown below:

	High Dose	**Low Dose**
Type 1	2.75 $\log_{10}$ $CCID_{50}$/5µl	1.75 $\log_{10}$ $CCID_{50}$/5µl
Type 2	6.0 $\log_{10}CCID_{50}$/5µl	5.0 $\log_{10}$ $CCID_{50}$/5µl
Type 3	4.5 $\log_{10}$ $CCID_{50}$/5µl	3.5 $\log_{10}$ $CCID_{50}$/5µl

- each dose is to be intraspinally inoculated into 16 male and 16 female mice;
- the doses inoculated are within the specified ranges, as defined below, and meet other relevant criteria;
- randomization of mice to doses, cage locations and inoculation order are carried out as described in Appendix 1.

The following sections provide some background to the statistical model, a decision model for the acceptance of the inoculum doses used in the test, specifications and examples for the statistical analysis of the paralysis data and the application of the pass/fail decision model

Analysis

If the experiment involves testing with more than one test vaccine, individual analyses of paralysis data are carried out comparing each test vaccine with the concurrent WHO (SO+2) Reference.

The analysis is also based on the assumption that the doses used in the test are the target doses specified. To confirm this, a decision model is applied to ascertain that, within accepted levels of

variation, the actual titres do not differ from the target doses.

Decision Model – Paralysis Data.

STEP 1. Check the paralysis proportions for the reference. If the combined (male and female) proportion at the high dose > 0.95 or the combined proportion at the low dose < 0.05 the reference data are regard as questionable. The experiment must be repeated (GO TO STEP 6). Otherwise GO TO STEP 2.

STEP 2. Check convergence of the model. If there is convergence apply the maximum likelihood procedure to the logistic regression model to test for a vaccine dose interaction.

- If there is convergence but the test for interaction cannot be performed due to observed paralysis rates for the test vaccine at 0.0 or 1.0, then GO TO STEP 3.
- If there is convergence and the test for interaction is not significant ($p > 0.05$), then GO TO STEP 4.
- If there is no convergence or the test for interaction is significant then apply the pass/fail criteria at each dose. The test vaccine passes if the vaccine passes at both doses (i.e. log odds ratio < L1). The test vaccine fails if the vaccine fails at both doses (i.e. log odds ratio > L2). All other outcomes will require a re-test (i.e. GO TO STEP 6).

STEP 3. If the procedure does not provide a valid test of interaction due to observed paralysis rates for the test vaccine at 0.0 or 1.0 then apply the criteria below:

- If both doses of the test vaccine produce legitimate 0.0 paralysis rates then the vaccine is accepted.
- If a 0.0 paralysis rate only occurs for the low dose and the high dose paralysis rate is lower than that of the corresponding reference then the test vaccine is accepted, otherwise proceed to STEP 5.
- If the test vaccine has paralysis rates of 1.0 at both doses, the test vaccine fails.
- If the test vaccine has a paralysis rate of 1.0 only at the high dose, then the decision process is applied to the combined results for both doses and also to the log odds ratio for the low dose. The vaccine is required to pass the decision criteria for both the combined estimate of the log odds ratio and the estimate at the low dose.

STEP 4. Check the dose effect. If the dose effect is significant ($p<0.05$, one-tailed test), GO TO STEP 5. If the dose effect is not significant check for vaccine effect. If vaccine effect is significant ($p < 0.05$), GO TO STEP 5. If vaccine effect is not significant, GO TO STEP 6.

STEP 5. Calculate the Log Odds Ratio.

- If Log Odds Ratio <= L1 Vaccine passes
- If L1 < Log Odds Ratio < L2 GO TO STEP 6
- If Log Odds Ratio => L2 Vaccine fails

As laboratories will not have established their own values for L1 and L2 the values initially set from the collaborative studies are given below. These are the values that should be used for the assessment of the training and implementation panels used in the procedures outlined in Appendix 3.

	L_1	L_2
Type 1	0.734	1.037
Type 2	0.665	0.940
Type 3	0.645	0.913

STEP 6. If a pass/fail decision is not reached in STEPS 1-5 and a repeat experiment is required, the decision process will be applied to either the pooled data from the two experiments or the data from the second experiment alone. The analysis depends on the circumstances which led to the retest:

- If the retest was initiated due to a technical problem in the first experiment or due to a lack of validity of the reference profile (e.g. failed Step 1 or Step 4) then STEPS 1-5 must be repeated using the tests and estimates from the second experiment alone.
- If the retest was initiated because the Log Odds Ratio is between L1 and L2 or because of a problem with the test profile (e.g. lack of convergence due to test paralysis rates near 1.00) then STEPS 1-5 must be repeated using the pooled data from the first and second experiment.

The test vaccine must satisfy the above criteria in order to pass. There will be no provision for additional retesting. If, after retesting, the log odds ratio for the test vaccine exceeds L_1 in any of the above STEPS, it is deemed to have failed. If a pass/fail decision cannot be reached because of problems with the reference (i.e. Paralysis rates <0.05 or >0.95, or no significant dose effect for the reference) then the mouse test must be re-established before any further testing is carried out.

Acceptance and Rejection Limits – Starting Values and Updating Values

In a laboratory establishing the current test, the absence of established historical reference data means that limits cannot initially be set. In this instance the limits shown above are used, these are in turn derived from the following historical values (obtained from collaborative studies) for the WHO (SO+2) reference materials *(1)*:

	Females Low Dose	Females High Dose	Males Low Dose	Males High Dose
WHO (SO+2)/I	0.162	0.785	0.133	0.705
(Phase 5, 1.75 and 2.75)	(13/80)	(62/79)	(10/75)	(55/78)
WHO(SO+2)/II	0.157	0.529	0.385	0.818
(Phase 5, 5.0 and 6.0)	(30/191)	(101/191)	(74/192)	(0.157/192
WHO(SO+2)/III	0.167	0.522	0.310	0.701
(Phase 4, 3.5 and 4.5)	(34/204)	(106/203)	(64/205)	(141/201)

As each experiment is successfully completed within a laboratory (including experiments completed during the training and implementation procedures) its reference vaccine paralysis data is pooled with the above data and limits are updated following the procedure outlined below

Updating Acceptance and Rejection Limits

This section provides formulas which are used to calculate the acceptance limit (L_1), the rejection limit (L_2) and the approximate probabilities of passing or failing vaccines. The symbols used in the formulas are defined below:

i denotes the dose/gender group:
1= females low dose, 2= males low dose
3= females high dose, 4= males high dose

P_{1i} = reference paralysis proportions in group i
P_{2i} = test vaccine paralysis proportions in group i
$Q_{1i} = 1 - P_{1i}$
$Q_{2i} = 1 - P_{2i}$
R_i = Odds Ratio in group I
L_i = Log Odds Ratio in group i
n_{1i} = sample size for reference in group i
n_{2i} = sample size for test in group i

The log odds ratio for subgroup 'i' is:

$$L_i = \log(R_i) = \log \frac{P_{2i} Q_{1i}}{Q_{2i} P_{1i}} .$$

The common log odds ratio for the four groups is:

$$L = \frac{\sum w_i L_i}{\sum w_i}$$

with a standard error of

$$s.e. = \frac{1}{\sqrt{\sum w_i}}$$

where

$$\frac{1}{w_i} = \frac{1}{n_{1i} P_{1i} Q_{1i}} + \frac{1}{n_{2i} P_{21} Q_{2i}} .$$

L is approximately normally distributed. The probability that the estimated Log Odds Ratio in an experiment will exceed a value of C is approximately:

Prob = Pr(Z > (C-L) / s.e.)

where Z is the standard normal deviate. The acceptance limit L_1 was selected so that a test vaccine which is equivalent to the reference will have a 0.95 probability of passing. That is,

$L_1 = 1.645$ s.e.

where 1.645 is the 5% standard normal deviate . The rejection limit L_2 was selected so that a test vaccine which is equivalent to the reference will have a 0.01 probability of failing. That is,

$L_2 = 2.326$ s.e..

For the purpose of calculating the starting limits, the historical reference paralysis rates were substituted for the reference probabilities, P_{1i} , in the above equations. For any specified odds ratio, R, test vaccine paralysis proportions, P_{2i} ,can be derived as follows.

Given the P_{1i} and a common odds ratio R we have

$$\frac{P_{2i}}{Q_{2i}} = R\frac{P_{1i}}{Q_{1i}}$$

$$\Rightarrow P_{2i} = R\frac{P_{1i}}{Q_{1i}}\left(1 - P_{2i}\right)$$

$$\Rightarrow P_{2i} = \frac{R\frac{P_{1i}}{Q_{1i}}}{1 + R\frac{P_{1i}}{Q_{1i}}}$$

These formulas were used to generate the acceptance/rejection limits shown in the table above, and can be used to calculate probabilities of rejection or acceptance for any specified value of R.

The reference paralysis rates provided above were used as starting reference values for laboratories which did not have an established reference database with the official methodology.

These reference values should be regularly updated. After each successful test with the reference the results of the test are added to the historical database to obtain revised estimates of P_{1i}. An example of the calculation of the revised historical reference paralysis proportions after a first experiment for Type 3 is:

Group	Initial Proportions	First Experiment	Updated Proportions
Females dose 3.5	0.167 (34/202)	2/16	0.164 (36/218)
Males dose 3.5	0.310 (63/203)	4/16	0.306 (67/219)
Females dose 4.5	0.522 (107/205)	9/16	0.525 (116/221)
Males dose 4.5	0.701 (141/201)	13/16	0.710 (154/217)

The revised proportions are then substituted into the above formulas to obtain revised limits for this laboratory. For the above example the revised limits are L_1=0.648 and L_2= 0.917.

At the end of the Training and Implementation program (Appendices 2 and 3) a laboratory will have garnered data for each serotype from at least 5 separate tests. These data should be used to establish L_1 and L_2 values for the laboratory. These values should be updated with any subsequent data generated. It is recommended that laboratories use the data from the last 10 tests to recalculate values for L_1 and L_2.

Decision Model – Doses Used.

The values of L_1 and L_2 used for the pass/fail decision model outlined above are based on the paralysis rates observed for the WHO (SO+2) reference. As the paralysis rate in the test is dependent on the dose inoculated i.e. paralysis rates show a dose response across the range of inoculum titres used in the tests there is a need to ensure that the doses used are within the accepted limits of variability, as close as possible to the target doses and/or not significantly different between test vaccine and WHO (SO+2).

Acceptance of Doses Used- Reference Calibration

It is recommended that the dilutions used in a test be assayed for titre at least twice, i.e. once before use and once after use.

Data from each assay point should be pooled (with at least three replicates from each assay point) and the pooled data should meet the following criteria:

- precision of $\pm$ 0.3 $\log_{10}$ $CCID_{50}$/ml or better for the 95% confidence limits of the mean for each dose used
- mean titre should be within $\pm$ 0.3 $\log_{10}$ $CCID_{50}$ of the target dose.

Acceptance of Doses Used – Agreement with Doses of the WHO (SO+2) Reference

The doses used for the reference and for the test vaccine must be in agreement. Normal in-house procedures for ensuring the validity of the assay must be followed.

The following criteria should be applied:

- precision of $\pm$ 0.3 $\log_{10}$ $CCID_{50}$/ml or better for the 95% confidence limits of the mean for each dose used

- mean titre of the in-house reference included in the assay should be within ± 0.3 $\log_{10}$ $CCID_{50}$ of the established value.
- mean titre of the each dose of the WHO(SO+2) standard should be within ± 0.3 $\log_{10}$ $CCID_{50}$ of the target dose
- mean titer of each vaccine dose should be within ± 0.3 $\log_{10}$ $CCID_{50}$ of the equivalent dose for the WHO(SO+2)

Worked example: In a type 3 test the titre of the WHO reference is found to be 6.7 and 5.7 $\log_{10}$ $CCID_{50}$/ml for the 4.5 and 3.5 $\log_{10}$ $CCID_{50}$/5µl doses respectively with 95% confidence limits of 0.2 and 0.3 $\log_{10}$ $CCID_{50}$/ml. The titre of the in-house reference is found to be 8.2 $\log_{10}$ $CCID_{50}$/ml compared to an assigned value of 8.4 $\log_{10}$ $CCID_{50}$/ml. The mean titres for the vaccine are 6.9 and 5.8 $\log_{10}$ $CCID_{50}$/ml for the 4.5 and 3.5 $\log_{10}$ $CCID_{50}$/5µl doses respectively. Using the criteria above, these doses are acceptable:

- the 95% confidence intervals of the estimated titres are within the ± 0.3 $\log_{10}$ $CCID_{50}$/ml specified
- the mean titre of the in-house reference is within ± 0.3 $\log_{10}$ $CCID_{50}$ of the assigned value
- the 4.5 and 3.5 $\log_{10}$ $CCID_{50}$/5µl doses correspond to target titres of 6.8 and 5.8 $\log_{10}$ $CCID_{50}$/ml and the titres of the WHO(SO+2)/III doses are within the specified limit (± 0.3 $\log_{10}$ $CCID_{50}$) of these target titres
- the titres for the vaccines doses are both within the specified limit (± 0.3 $\log_{10}$ $CCID_{50}$) of the doses for the corresponding WHO(SO+2)/III doses.

Test dilutions should only be inoculated in mice if doses have been found acceptable. If the titre of any of the vaccine doses used is found to be out of specification when titred after the test, a repeat of the back-titration is possible and the results of both titrations could be pooled and reassessed, but this should always be in agreement with the standard retest policy. If the titre of any of the vaccine doses used is found to be outside of the limits noted above after retesting, a statistical review of the data should be performed to decide whether the TgmNVT needs repeating and new dilutions prepared.

Appendix 5: Monitoring of data from the Transgenic Mouse Neurovirulence Test (TgmNVT)

The Transgenic Mouse Neurovirulence Test (TgmNVT) generates a large amount of data that need to be monitored to ensure the performance of the test remains consistent and that pass/fail decisions are consistent and reliable.

Each stage of the training and implementation process, as well as the subsequent testing program, needs to be monitored and documented for both individual inoculators and organizations. It is also possible that these data may be requested by the NCL or other relevant authorities.

The following provides an outline of the data that need to be monitored. Additional data may be requested by the NCL.

Dye Inoculation
Records should be kept of the performance of each inoculator for each test carried out during the training period (Appendix 2) and for any subsequent dye inoculations carried out e.g. those carried out after a break in inoculations (see Appendix 2 – Maintenance of Competence)

Clinical scoring
A log of the clinical scoring should be kept for each observer. In the case of a long period of inactivity (> 6 months) measures should be taken to reconfirm competence in observation skills before a test scoring session is undertaken and these should be documented.

Titration of References
The TgmNVT relies on the accurate estimation of the titre of virus inocula used during the test and on the match of these titres to the nominal doses used in the test. The titre of the titration reference should be monitored to ensure the consistency of the assay.

Traumatic Paralysis
Records should be kept of the number animals that suffer from traumatic paralysis as a result of the inoculation. If ≥5% of inoculated animals suffer traumatic paralysis, the inoculation technique is inadequate and actions should be taken to improve performance.

Paralysis Rates for the WHO Reference
The paralysis rates for the WHO reference should be monitored for each dose and each inoculator and records maintained.

LOR for vaccine batches
L-values should cover all tests conducted by the laboratory, but to detect variability between operators, laboratories may wish to additionally calculate L-values for individual operators.
The correlation of LOR values and the results obtained for the MAPREC or other in vitro tests of neurovirulence may additionally be monitored for each operator to increase consistency.

Authors

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Further changes were made, taking into consideration comments received during the public consultation along with the WHO/BS/2012.2185, and following the review by the 63rd Expert Committee on Biological Standardization,15-19 October 2012, resulting in the present document.

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Médicament (ANSM), France; Peter Rigsby, NIBSC, UK; Vicki Bockstal and Barbara Sanders, Crucell (Johnson & Johnson), The Netherlands; Amélie Castiaux, Thierry Monmart and Stéphanie Van Brabant, GlaxoSmithKline Biologicals, Belgium; Angela Gomersbach, Danielle Lankveld, Hans Strootman and Susan Van Beem, Intravacc, The Netherlands; Laurent Fanchon and Davy Vidon-Buthion, Sanofi Pasteur, France. This revision includes improvements of some text in the decision model for statistical analysis in appendix 4, to provide clear guidance.

The current version (Version 8, 2021) was prepared by Ms. Laura Stephens and Mrs. Lisa Johnson, National Institute for Biological Standards & Control (NIBSC) and Dr Catherine Milne, European Directorate for the Quality of Medicines and Health Care, Council of Europe (EDQM), France, taking into consideration requests and agreements at the NIBSC/EDQM annual workshops on Training and Implementation of the Transgenic Mouse Test for Oral Polio vaccine held at NIBSC,UK between 2018 to 2020. Workshop participants include experts from industry and OMCLs that actively perform or review performance of the testing. This revision includes advice on the injection, preparation of samples for reading, recording and interpreting the results in Appendix 2 with a view to provide additional critical details regarding the training of inoculators using ink injections.

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